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Research Article

An efficient iDNA method for surveying rainforest mammals with carrion flies

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Abstract

Metabarcoding of vertebrate DNA derived from invertebrates (iDNA) is a promising method for monitoring of mammal assemblages. However, optimization of field and laboratory methods for efficient and economical sampling and analysis is needed. We used metabarcoding of carrion flies to survey for mammals at Los Amigos Biological Station in the Peruvian Amazon. We used an extraction-free, direct PCR approach to metabarcode vertebrate DNA, whereby buffer from tubes that contained flies for 3-5 hours was used directly in PCR, negating the need for labor-intensive DNA extraction. We also examined two different pooling methods to investigate the efficacy of pooling multiple fly samples to economize sequencing of large numbers of samples. Metabarcoding detected 27 mammalian taxa from 9 orders and 18 families plus four anuran taxa from three families from 240 carrion flies collected over 3 days. Pooling of buffer from multiple flies prior to PCR resulted in failure to detect many species compared to sequencing of individual flies. However, pooling of PCR product after an initial PCR from individual flies, but prior to indexing, resulted in species detection nearly identical to sequencing of individual flies. These methodological findings will contribute to the feasibility of iDNA metabarcoding for vertebrate monitoring and biodiversity conservation.

Key words: Amazon basin, eDNA, Environmental DNA, biodiversity monitoring, metabarcoding

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Introduction

Environmental DNA (eDNA) is a powerful tool for biodiversity monitoring (Bohmann et al. 2014; Ruppert et al. 2019). Historically, vertebrate eDNA research focused primarily on aquatic species; however, eDNA methods have more recently been adapted for the study of terrestrial vertebrates (Broadhurst et al. 2025; Newton et al. 2025). In terrestrial systems, invertebrate-derived DNA (iDNA) methods using invertebrates such as carrion flies (Calvignac-Spencer et

al. 2013; Rodgers et al. 2017), dung beetles (Drinkwater et al. 2021; Saranholi et al. 2024) and leeches (Ji et al. 2022) allow for passive surveillance of terrestrial vertebrate communities. By acquiring traces of vertebrate DNA through interactions with feces, carcasses, blood, or dead skin from live animals, these invertebrates can assist in the monitoring of terrestrial vertebrate assemblages. This method allows detection of vertebrate species presence without direct sightings or captures and offers an effective and non-invasive approach for surveys in tropical rainforest ecosystems (Rodgers et al. 2017) where high biodiversity, cryptic species, remote landscapes, and a rich canopy fauna may limit the efficacy of camera traps or other survey methods.

Although iDNA metabarcoding has been used effectively for vertebrate sampling (Calvignac-Spencer et al. 2013; Drinkwater et al. 2021; Ji et al. 2022; Rodgers et al. 2017; Saranholi et al. 2024; Schubert et al. 2015), methodological advancements to increase efficiency are needed (Jahan et al. 2025). DNA extraction is often one of the most time-consuming steps in eDNA or iDNA metabarcoding laboratory workflows. For carrion fly metabarcoding, past methods required macerating whole flies into a slurry for DNA extraction (Calvignac-Spencer et al. 2013; Rodgers et al. 2017) or dissecting fly digestive tracts (Lee et al. 2016), processes that demand substantial effort and cost. This study used carrion fly iDNA to survey mammalian diversity in the Amazon rainforest of Los Amigos Biological Station, Madre de Dios, Peru through iDNA metabarcoding by instead using an efficient, extraction-free direct PCR approach.

The aim of this study was to test methods to improve the efficiency of field and laboratory workflows for carrion fly iDNA metabarcoding. We tested a field method of netting carrion flies directly off a bag of pork bait to avoid having to catch flies in traps. We also used an extraction-free, direct PCR approach (Srivathsan et al. 2023) for next generation sequencing of mammal DNA derived from carrion flies. This method amplifies DNA regurgitated or deposited by flies inside collection tubes directly from the tube without need for formal extraction or purification. This direct PCR method greatly reduces laboratory processing time by negating the need for maceration or dissection, and DNA extraction altogether. Additionally, we compared two different methodologies for pooling fly samples during library preparation to investigate the most efficient approach for large-scale biodiversity assessments where sequencing of individual flies is cost prohibitive.

Methods

Study site

Los Amigos Biological Station (LABS; also known as CICRA in previous literature), is located at 12°33'40.0"S, 70°05'46.4"W in the Amazon Basin at an elevational range from 225 to 296 meters above sea level in the Madre de Dios region of southeastern Peru (Fig. 1). The sampling area was at the convergence of the Los Amigos and Madre de Dios rivers. This area includes a range of habitats including mature old growth forest on terra firme terraces of ancient, weathered soils of low pH, as well as both mature and successional forest found on the more fertile alluvial floodplains that are annually flooded, where there are dense stands of *Guadua* bamboo and *Mauritia flexosa* palm swamps.

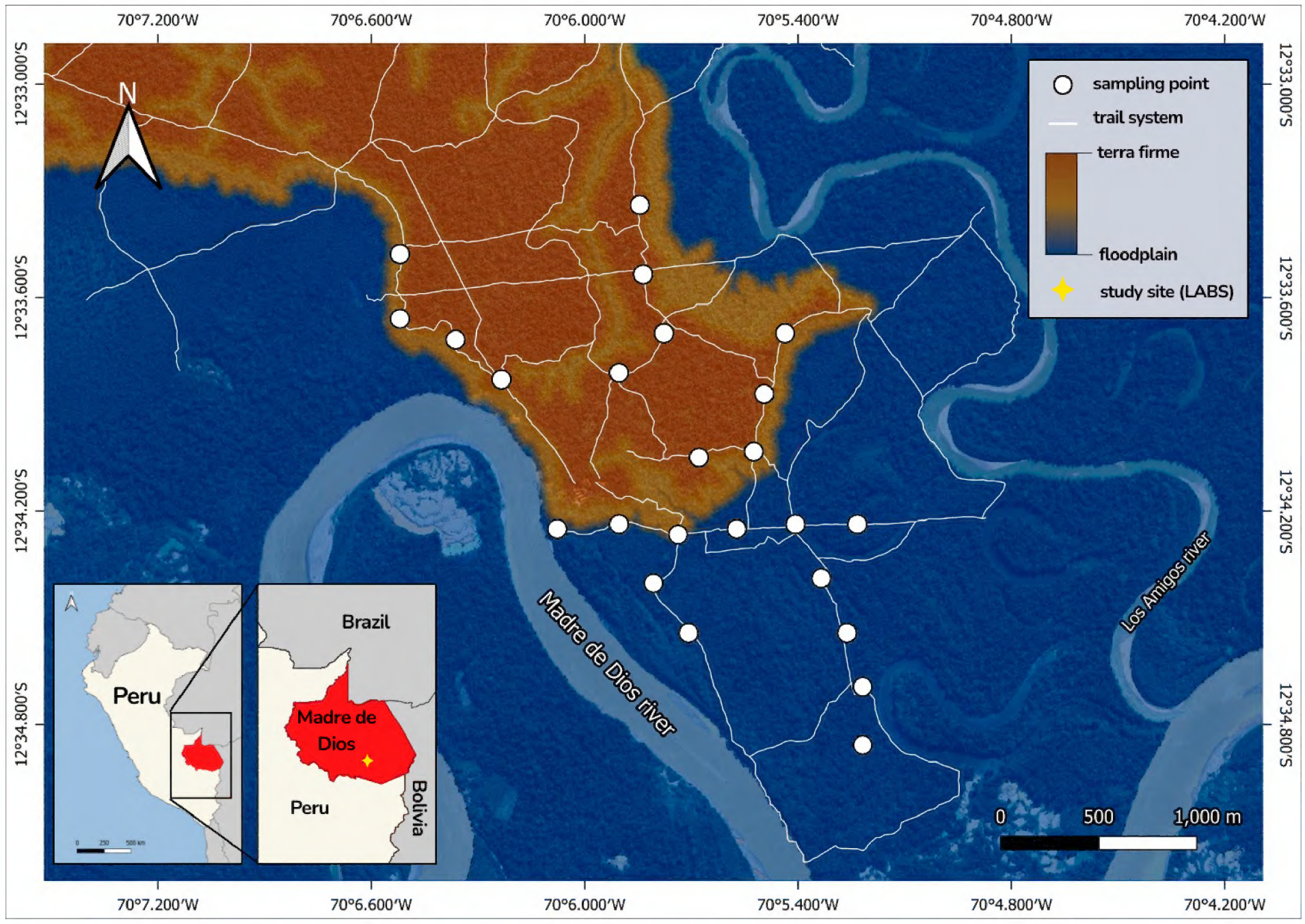

Figure 1. Map of study site for collection of carrion flies for iDNA metabarcoding to detect rainforest mammals at Los Amigos Biological Station, Madre de Dios, Peru.

The region has a tropical humid climate with a rainy season from November to February and a dry season from June to September. Annual rainfall ranges between 2700 and 3000 mm, and the mean temperature ranges from 21 °C to 26 °C. The region supports a diverse mammalian community that is currently unhunted and is typical of intact lowland rainforests in the western Amazon.

Carrion fly collection

We collected carrion flies at 24 sampling sites by netting them directly off a white plastic grocery bag loosely tied at the top, which contained an open Ziplock bag filled with rotting pork. We left the pork to rot under the sun for at least 24 hours prior to sampling. We conducted fly sampling from November 9–11, 2023. At each site, we netted 10 flies and placed each directly into a sterile 2 ml or 1.5 ml snap-cap microcentrifuge tube. After collecting 10 flies from one site, we moved 300 meters farther along the trail to select the next site. We repeated this process four times each morning and afternoon, collecting a total of 240 flies across 24 locations from six trail segments, three in floodplain habitat and three in terra firme habitat (Fig. 1).

We brought captured flies to the field laboratory and left them in tubes at ambient temperature for 3–5 hours to allow time for flies to regurgitate or deposit mammal DNA in tubes. Afterward, we removed the flies from tubes and placed flies in whirl-Pak bags with 90% ethanol for later morphological species

identification. We then added 4 to 5 3-5 mm non-sterile premium blue indicating silica gel desiccant beads (Dry & Dry; Brea, California, USA) to each tube to dry the interior and stored the tubes in a –20 °C freezer until transport to the molecular laboratory. Before transport, we removed and discarded the silica gel beads, then transported the dry tubes containing residual DNA regurgitated or deposited by flies to the Molecular Ecology Laboratory at Utah State University, where we placed them in a –70 °C freezer (Fig. 2A).

Laboratory methods

We used an extraction-free, direct PCR approach for DNA amplification (Fig. 2B). For each fly sample, we added 40 µL of direct PCR lysis buffer (from the Platinum™ Direct PCR Universal Master Mix, Invitrogen, Carlsbad, CA) and 1.2 µL of proteinase K to the microcentrifuge tube. We vortexed the tubes for two minutes, incubated them at room temperature for ten minutes, and then heated them on a 98 °C block for one minute before cooling to room temperature, following the manufacturer's lysis protocol. We then centrifuged the samples for one minute and added the resulting buffer template solution directly to the PCR reactions.

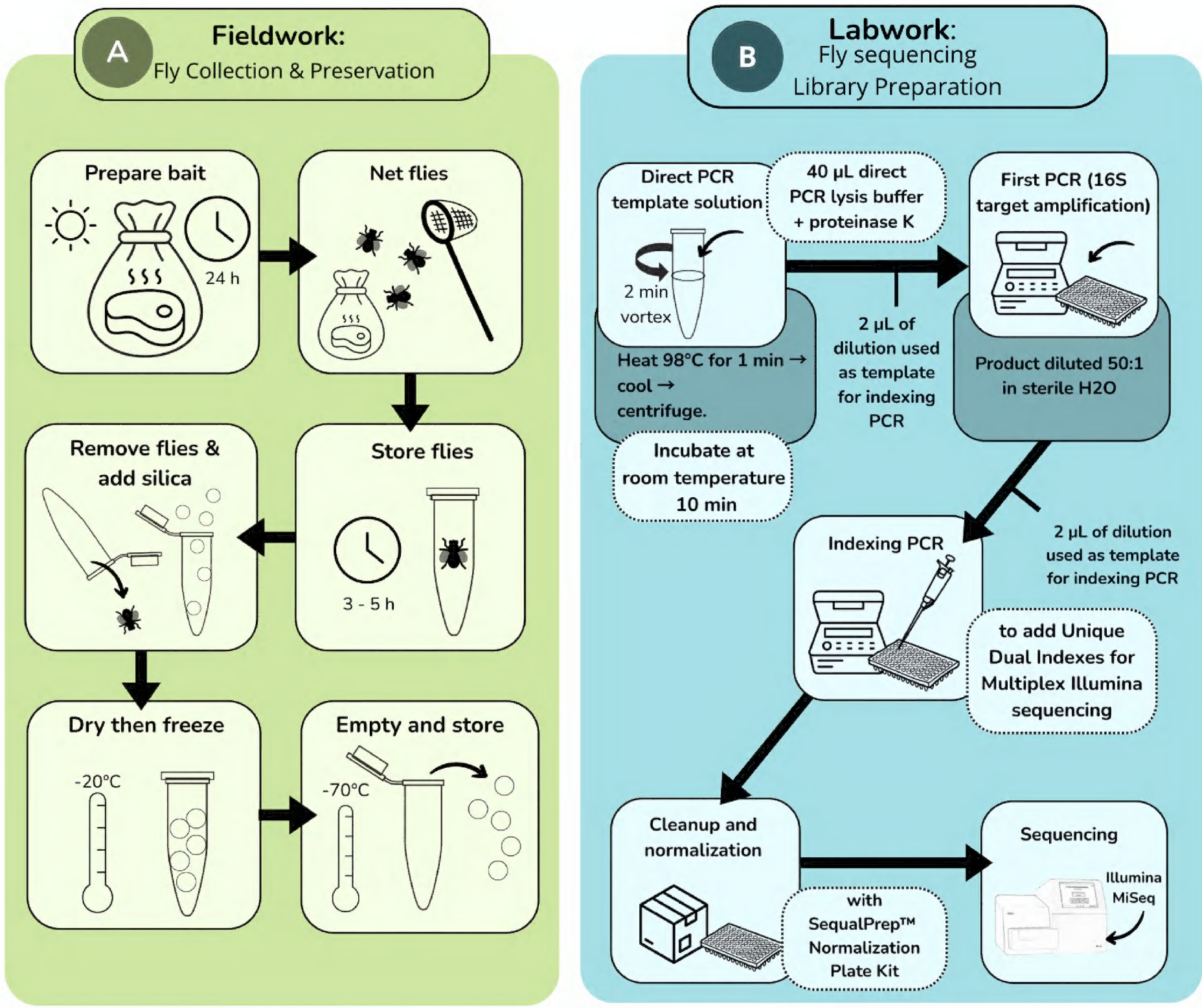

Figure 2. Workflow for a) carrion fly collection field work and B) laboratory work for sequencing of individual carrion flies for detection of rainforest mammals with iDNA from Los Amigos Biological Station, Madre de Dios, Peru.

PCR reactions contained 10 µL Platinum™ Direct PCR Universal Master Mix (Invitrogen, Carlsbad, CA), 4 µL Platinum™ GC Enhancer, 0.2 µM each of forward and reverse primers (16Smam1-To and 16Smam2; Taylor 1996; Table 1) containing Illumina sequencing adapters, 1 µM (5X) human blocking primer (16Smam-blkhum; Table 1), 1 µM (5X) domestic pig *Sus scrofa* blocking primer (16Smam-blkpig; Table 1), and 2 µL of direct PCR lysis buffer template solution from sample tubes for a final PCR reaction volume of 20 µL. PCR cycling conditions were: initial denaturation at 94 °C for 10 min, 40 cycles of 94 °C for 15 sec, 60 °C for 15 sec, and 68 °C for 20 sec, followed by a final elongation of 68 °C for 5 minutes. Each PCR run included a no-template control of sterile molecular grade water, a lysis buffer negative control, and a positive control containing 0.1 ng of DNA from bobcat *Lynx rufus*, a species that does not occur in South America. All negative and positive controls were indexed and sequenced. All pre-PCR steps and PCR setup were conducted under a laminar flow PCR hood with UV sterilization in an area physically separate from post PCR work.

Table 1. Primers used for metabarcoding of carrion fly-derived eDNA samples. The 1st and 2nd primers are forward and reverse primers respectively. The 3rd and 4th primers are blocking primers. These primers produce an amplicon of approximately 138 base pairs including primers.

Primer name	Primer sequence 5' to 3'	Citation
16Smam1-To	CGGTTGGGGYGACCTCGGA	Modified from Taylor 1996
16Smam2	GCTGTTATCCCTAGGGTAACT	Taylor 1996
16Smam-blkhum	CGGTTGGGGCGACCTCGGAGCAGAACCC - spacerC3	Boessenkool et al. 2012
16Smam-blkpig	CGGTTGGGGTGACCTCGGAGTACAAAAAAC - spacerC3	Calvignac-Spencer et al. 2013

We diluted PCR products from initial amplification at 50:1 in sterile H_2O and used dilutions as template for the indexing PCR. We performed indexing PCR to add unique dual indexes to each sample for multiplexing. The Unique Dual Indexing (UDI) primers (Integrative DNA Technologies) included a complementary Illumina flow-cell adapter sequence and an 8 bp unique dual index on each end (forward and reverse) to allow for unique sample identification in multiplexed libraries. Indexing PCR reactions contained 10 µl of Amplitaq Gold DNA polymerase (Thermo Fisher Scientific), 0.5 uM of forward and reverse UDI indexing primers, and 2 µl of diluted PCR product from the first PCR in a total reaction volume of 20 µl. PCR cycling conditions for the indexing PCR were: initial denaturation at 95 °C for 10 min, 15 cycles of 95 °C for 15 sec, 50 °C for 30 sec, and 72 °C for 30 sec, followed by a final elongation of 72 °C for 7 minutes. Following the indexing PCR, we cleaned and normalized PCR products using a SequalPrep™ normalization plate (Thermo Fisher Scientific) following the manufacturer's protocol.

Pooling strategies

We conducted PCR and sequencing on all fly sample tubes individually. In addition, we tested two pooling methods for combining individual fly samples into pooled samples for sequencing (Fig. 3). Because sequencing of individual flies may be prohibitively expensive for large datasets, we compared results from pooled samples with results from individual flies to see if pooling

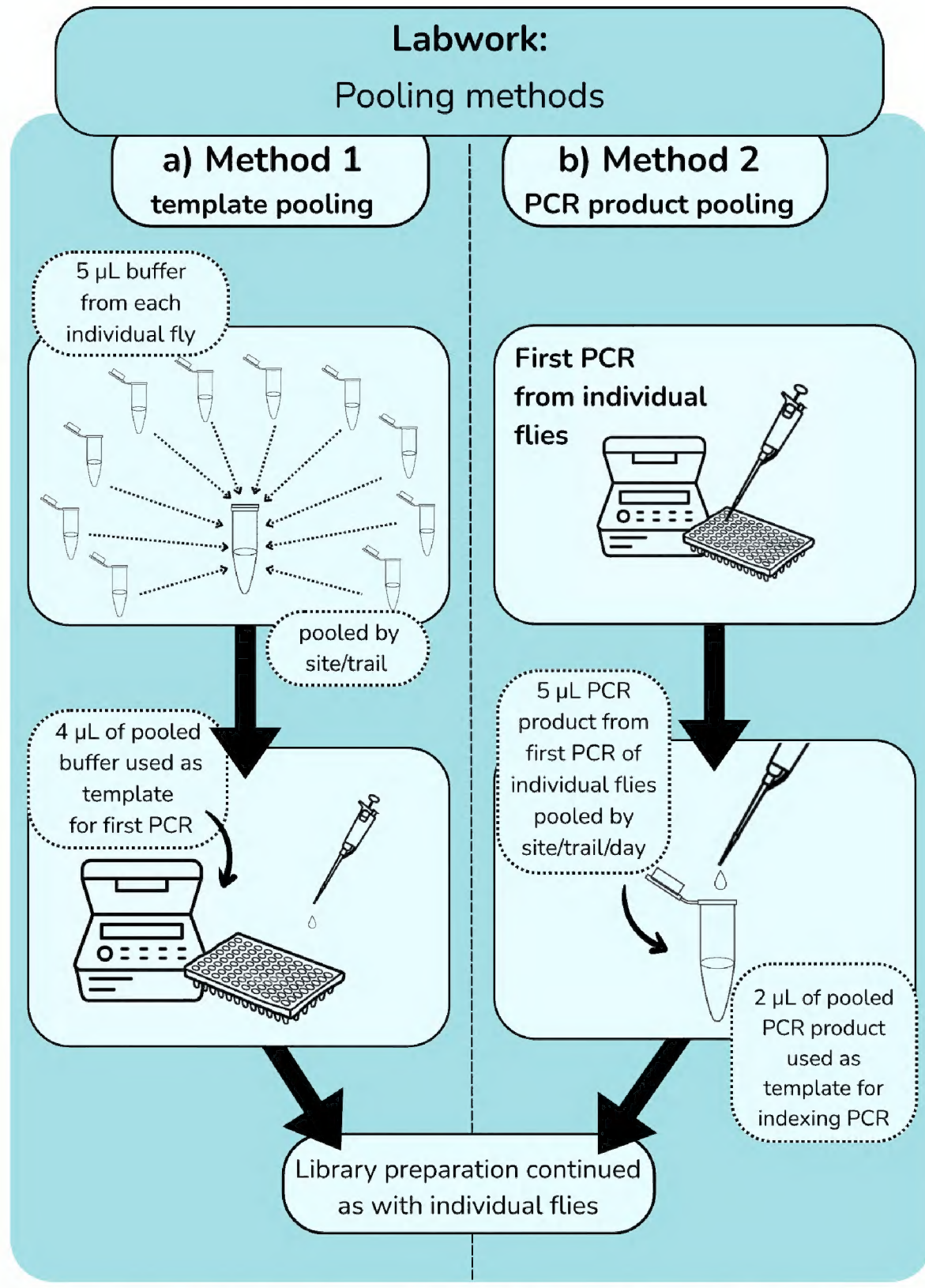
Labwork:
Pooling methods
a) Method 1
template pooling
b) Method 2
PCR product pooling
5 µL buffer from each individual fly
pooled by site/trail
4 µL of pooled buffer used as template for first PCR
First PCR from individual flies
5 µL PCR product from first PCR of individual flies pooled by site/trail/day
2 µL of pooled PCR product used as template for indexing PCR
Library preparation continued as with individual flies

Figure 3. Two methods tested for pooling of samples to economize metabarcoding of iDNA from carrion flies for the detection of rainforest mammals from Los Amigos Biological Station, Madre de Dios, Peru.

of samples led to loss of species detected. For the first method (hereafter referred to as template pooling), we pooled 5 µL of lysis buffer template from each of the 10 flies collected at each sampling site. Additionally, we pooled 5 µL of lysis buffer template from each of the 40 flies collected from the same trail segment (Fig. 3a). The initial PCR was conducted using 4 µL of pools as template, after which the library preparation protocol was identical to that from individual flies. For the second pooling method (hereafter referred to as PCR product pooling), we pooled PCR product from the initial PCR of individual flies prior to indexing. For this method, we pooled 5 µL of PCR product from each of the 10 flies collected at each sampling site. Additionally, we made pools of PCR products from each of the 40 flies collected from the same trail, and from each of the 80 flies collected on each day of sampling (Fig. 3b). We then diluted this pooled PCR product 10:1 in sterile H_2O and added 2 µL of pooled product to indexing PCR as above.

We sequenced samples in three separate libraries. The first library included 80 individual fly samples along with 105 samples from a separate project.

The second library contained the remaining 160 individual fly samples and the samples generated from template pooling. The third library consisted exclusively of samples from PCR product pooling. We included positive and negative controls in all three libraries. Each library was sequenced on an Illumina MiSeq using a 300-cycle v2 Micro reagent kit at the Center for Integrative Biosystems Sequencing Core at Utah State University.

Bioinformatic processing of MiSeq sequence data proceeded as follows. After demultiplexing, we removed primer sequences with CUTADAPT v. 1.18 (Martin 2011). Next, data were filtered, denoised, paired-ends were merged, chimeras were removed with DADA2 (Callahan et al. 2016), and Amplicon Sequence Variants (ASV) were merged into Operational Taxonomic Units (OTUs) at 97% similarity with VSEARCH (Rognes et al. 2016), all within the QIIME2 environment (Bolyen et al. 2019) version 2023.5. For taxonomic assignment of OTUs, we compared two methods: BLAST (Altschul et al. 1990) and a naïve Bayes classifier (Pedregosa et al. 2011) implemented within the QIIME2 environment. For BLAST, we retrieved the top 5 hits for each OTU, and taxonomy was assigned subjectively based on percent similarity of top hits (Table 2) with taxa known from the study area based on local expertise and a published species checklist from a nearby area (Payne et al. 2024). For taxonomic assignment with the naïve Bayes classifier, we used the lrRNA (16S) reference database containing unique haplotypes from MIDORI 2 version GB265 (Leray et al. 2022). We first extracted our specific amplicon of interest from the database using our primer sequences, and then we trained a naïve Bayes classifier using scikit-learn to assign taxonomy within QIIME2.

Because the PCR product pooling method had approximately 9 times greater sequencing read-depth than the template pooling method (see results), we conducted a rarefaction of reads from PCR product pooling to see if reducing read depth with rarefaction changed results. We carried out rarefaction in QIIME2, with a reduced sampling depth of 10,245 reads per sample to match read-depth of template pooling samples.

For OTUs that could not be assigned with high confidence at the species level with either of our classification methods, in some cases we subjectively assigned a “presumed species” based on local expertise of the known mammal community, and a species checklist from a nearby area (Payne et al. 2024). We did this when an OTU was assigned only to genus with confidence, but when only one species in the given genus is known from the study area. In two cases, we also subjectively assigned “presumed species” to OTUs only assigned at the family level, but that we believe belong to taxa from our study area that lack reference sequences (Table 2).

Results

Carrion fly collection

We collected 240 carrion flies from 24 locations along six trails. Because of the difficulty of identifying carrion flies to species based on morphology, flies were in most cases only identified to family. The most common families collected were Calliphoridae, Sarcophagidae, Mesembrinellidae, and Tachinidae representing 53%, 35%, 6%, and 3% of total flies collected respectively. We also collected a few flies from the families Syrphidae (n = 3) and Muscidea (n = 3).

Table 2. Species detected from DNA metabarcoding of carrion flies from Los Amigos Biological Station, Madre de Dios, Peru, based on two taxonomic assignment methods, naïve Bayes, and BLAST.

Class	Order	Family	naïve Bayes species	naïve Bayes confidence	BLAST top hit species	BLAST match %	Presumed species	Species common name
Mammalia	Artiodactyla	Cervidae	*Odocoileus* sp.	0.857	*Mazama americana*	100.0	*Mazama americana*	Red Brocket Deer
Mammalia	Artiodactyla	Cervidae	*Passalites nemorivagus*	0.937	*Passalites nemorivagus*	98.9	*Passalites nemorivagus*	Amazonian Brown Brocket
Mammalia	Artiodactyla	Tayassuidae	*Catagonus wagneri*	0.988	*Catagonus wagneri*	93.5	*Tayassu pecari*	White-lipped Peccary
Mammalia	Artiodactyla	Tayassuidae	*Dicotyles tajacu*	1.000	*Dicotyles tajacu*	98.9	*Dicotyles tajacu*	Collared Peccary
Mammalia	Carnivora	Felidae	*Puma concolor*	1.000	*Puma concolor*	100.0	*Puma concolor*	Puma
Mammalia	Carnivora	Mustelidae	*Unknown*	1.000	*Gulo gulo*	96.7	*Eira barbara*	Tyra
Mammalia	Carnivora	Procyonidae	*Bassaricyon alleni*	0.984	*Bassaricyon alleni*	96.3	*Bassaricyon alleni*	Eastern Lowland Olingo
Mammalia	Carnivora	Procyonidae	*Nasua nasua*	1.000	*Nasua nasua*	97.4	*Nasua nasua*	South American Coati
Mammalia	Carnivora	Procyonidae	*Potos flavus*	1.000	*Potos flavus*	96.8	*Potos flavus*	Kinkajou
Mammalia	Chiroptera	Molossidae	*Molossus molossus*	0.923	*Molossus molossus*	98.9	*Molossus molossus*	Velvety Free-tailed Bat
Mammalia	Chiroptera	Phyllostomidae	*Artibeus planirostris*	0.988	*Artibeus planirostris*	100.0	*Artibeus planirostris*	Flat-faced Fruit-eating Bat
Mammalia	Chiroptera	Phyllostomidae	*Uroderma bilobatum*	1.000	*Uroderma bilobatum*	96.7	*Uroderma bilobatum*	Tent-making Bat
Mammalia	Cingulata	Dasypodidae	*Dasypus novemcinctus*	0.788	*Dasypus sabanicola*	100.0	*Dasypus* sp.	Naked-tailed Armadillo
Mammalia	Didelphimorphia	Didelphidae	*Didelphis marsupialis*	0.919	*Didelphis marsupialis*	100.0	*Didelphis marsupialis*	Common Opossum
Mammalia	Perissodactyla	Tapiridae	*Tapirus terrestris*	0.971	*Tapirus terrestris*	100.0	*Tapirus terrestris*	Lowland Tapir
Mammalia	Primates	Aotidae	*Aotus* sp.	1.000	*Aotus nigriceps*	100.0	*Aotus nigriceps*	Black-Headed Night Monkey
Mammalia	Primates	Atelidae	*Alouatta seniculus*	1.000	*Alouatta seniculus*	100.0	*Alouatta seniculus*	Red Howler Monkey
Mammalia	Primates	Atelidae	*Ateles chamek*	0.977	*Ateles chamek*	100.0	*Ateles chamek*	Black Spider Monkey
Mammalia	Primates	Cebidae	*Cebuella* sp.	0.974	*Cebuella pygmaea*	93.4	*Cebuella niveiventris*	Eastern Pygmy Marmoset
Mammalia	Primates	Cebidae	*Cebus unicolor*	0.999	*Cebus unicolor*	100.0	*Cebus cuscinus*	Shock-headed Capuchin
Mammalia	Primates	Cebidae	*Leontocebus fuscicollis*	1.000	*Leontocebus fuscicollis weddelli*	100.0	*Leontocebus weddelli*	Weddell's Saddle-back Tamarin
Mammalia	Primates	Cebidae	*Saimiri* sp.	1.000	*Saimiri boliviensis*	98.9	*Saimiri boliviensis*	Bolivian Squirrel Monkey
Mammalia	Primates	Cebidae	*Sapajus*	1.000	*Sapajus apella*	100.0	*Sapajus apella*	Tufted Capuchin
Mammalia	Rodentia	Cricetidae	*Oligoryzomys flavescens*	0.930	*Oligoryzomys flavescens*	86.4	*Oligoryzomys* sp.	Pygmy Rice Rat
Mammalia	Rodentia	Cuniculidae	*Cuniculus paca*	1.000	*Cuniculus paca*	100.0	*Cuniculus paca*	Lowland Paca
Mammalia	Rodentia	Echimyidae	*Mesomys hispidus*	0.985	*Mesomys hispidus*	95.7	*Mesomys hispidus*	Spiny Tree Rat
Mammalia	Rodentia	Erethizontidae	*Coendou insidiosus*	0.999	*Coendou insidiosus*	94.6	*Coendou* sp.	Prehensile-tailed Porcupine
Amphibia	Anura	Hylidae	*Dendropsophus koechlini*	1.000	*Dendropsophus koechlini*	100.0	*Dendropsophus koechlini*	Koechlin's Treefrog
Amphibia	Anura	Hylidae	*Scinax ictericus*	1.000	*Scinax ictericus*	100.0	*Scinax ictericus*	Tree frog species
Amphibia	Anura	Leptodactylidae	*Leptodactylus stenodema*	0.999	*Leptodactylus stenodema*	91.8	*Leptodactylus stenodema*	San Jose White-lipped frog
Amphibia	Anura	Microhylidae	*Ctenophryne geayi*	1.000	*Ctenophryne geayi*	100.0	*Ctenophryne geayi*	Brown Egg Frog

Carrion fly laboratory analysis

MiSeq sequencing of carrion fly samples from the first two libraries (individual flies and pool samples from template pooling) resulted in 3,965,439 reads, of which 2,547,579 reads were retained after quality filtering, denoising, and chimera removal, with a median of 9,956 reads per individual fly sample, and a median of 10,245 reads per pool sample. MiSeq sequencing of carrion fly pool samples from the third library (pooling method two) resulted in 3,280,546 reads, of which 3,086,005 reads were retained after quality filtering, denoising, and chimera removal, with a median of 92,385 reads per pool sample. These reads were clustered into 78 unique OTUs. After removal of OTUs identified by BLAST as belonging to human or non-target domestic species (*Sus scrofa*, *Bos taurus, Canis familiaris, and Felis catus*), the *Lynx rufus* positive control (which was found only in the positive control sample) and OTUs that could not be identified to the family level, 40 unique OTUs assigned to vertebrate species remained. Only human reads were found in the negative controls.

We assigned these 40 OTUs to 31 unique taxa (Table 2, Fig. 4) including eight primates, five carnivores (one felid, three procyonids, and one mustelid), four rodents, four artiodactyls, one perissodactyl, three chiropterans, one armadillo, one marsupial, and four anurans. Of these 31 unique taxa, 26 were assigned to a species known from the study area. Of the remaining 5 taxa, 3 were assigned only at the genus level and 2 at the family level. We subjectively assigned a “presumed species” to two OTUs that our classifiers could only assign objectively to the family level. In both cases, a reliable reference sequence for 16S for the species in question was not available in GenBank. However, in both cases, all other species from the given family known from the study area do possess a reliable reference sequence, leading us to believe that these OTUs belong to the “presumed species”. In one case, an OTU was identified by both classifiers as Chacoan peccary *Catagonus wagneri* (Table 2). The Chacoan peccary does not occur in Peru, and thus, it is not possible that the sequence originated from this species. However, the white lipped peccary *Tayassu pecari* is present at Los Amigos, but a reliable 16S reference sequence for this species is not available in GenBank. GenBank does possess a partial mitochondria genome for *T. pecari* (Accession: KY987556), but this genome is low quality, with many ambiguous bases in the region corresponding to our amplicon. *C. wagneri* is the most closely related extant species to *T. pecari* and is more closely related to *T. pecari* than to collared peccary *Pecari tajacu* (Parisi Dutra et al. 2017), the other peccary species present at Los Amigos. Thus, we presume this OTU belongs to white lipped peccary. In another case, one OTU was assigned by the naïve Bayes classifier only to the family Mustelidae, while BLAST returned a top hit at 96.7% to *Gulo gulo* or wolverine, with all 5 top hits assigned to species within the family Mustelidae, but none to species known from Los Amigos. Three of the four mustelid species known from Los Amigos have a 16S reference sequence available apart from the tayra *Eira barbara*. Thus, we presume this OTU belongs to *E. barbara*.

In total, we detected and taxonomically assigned vertebrate DNA from 106/240 (44.2%) individual flies (excluding human or domestic species). Among these 106 flies, 63 yielded DNA from a single species, 31 flies yielded

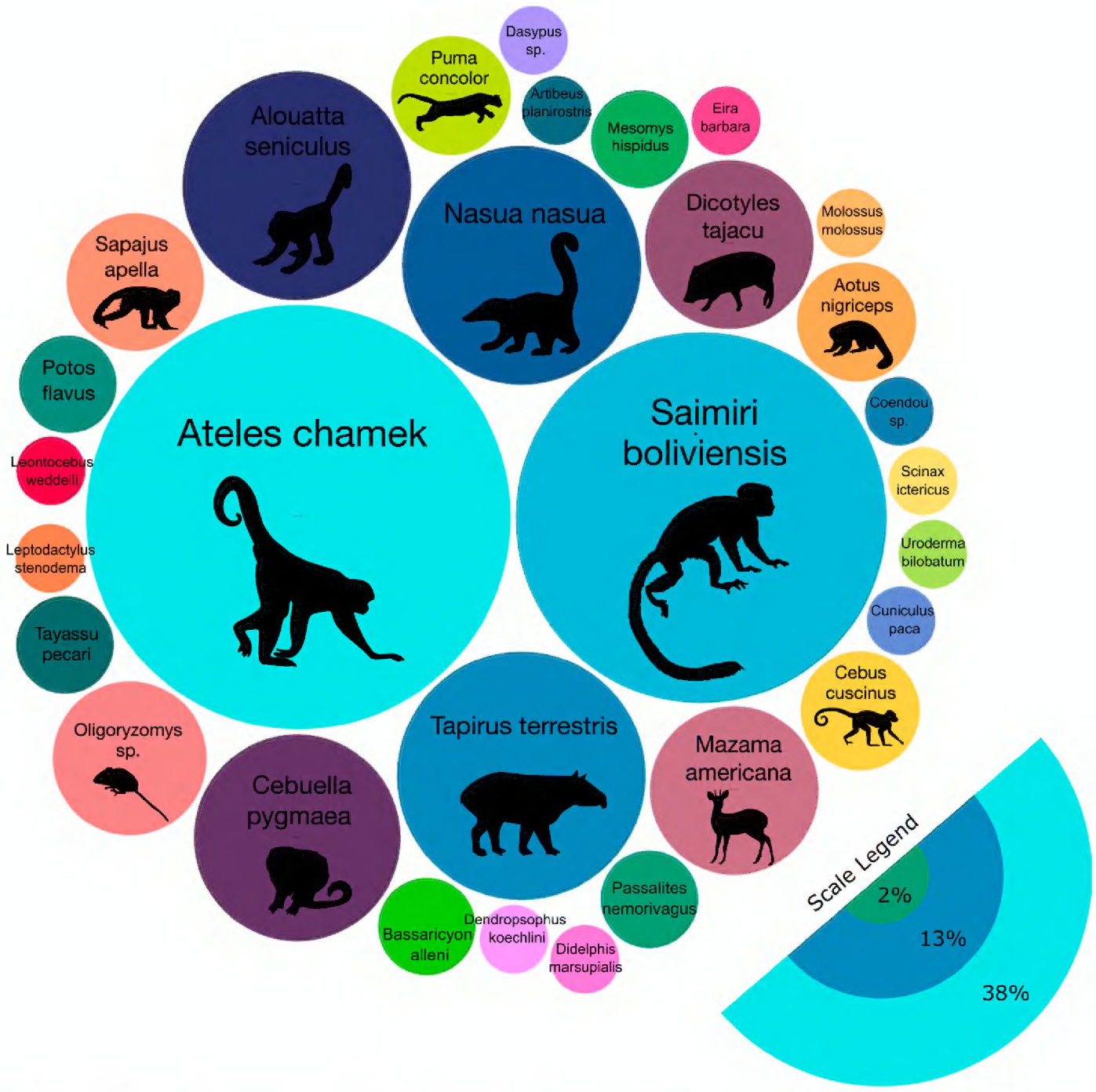

Figure 4. Visual representation of mammals detected by metabarcoding of carrion flies from Los Amigos Biological Station, Madre de Dios, Peru. Size of circles represents the percentage of flies containing vertebrate DNA belonging to each species (Table 3).

DNA from two species, ten flies yielded DNA from three species, one fly yielded DNA from four species, and one fly yielded DNA from five species. The mean number of flies yielding DNA per site was 4.0 (range 1-6). The mean number of species detected per site was 4.96 (range 1-8), with at least one vertebrate species detected from all 24 sites.

The 106 fly samples containing vertebrate DNA represented 5 Dipteran families: 67 (63%) from Calliphoridae, 31 (29%) from Sarcophagidae, three (2.8%) from Tachinidae, three (2.8%) from Mesembrinellidae, and two (1.8%) from Muscidae. This corresponds to 53% (67/126) of flies from Calliphoridae, 37% (31/84) of flies from Sarcophagidae, 37% (3/8) of the flies from Tachinidae, 67% (2/3) of the flies Muscidae, and 20% (3/15) of the flies from Mesembrinellidae containing vertebrate DNA. No vertebrate DNA was detected from the three flies from the family Syrphidae.

Primates were the most commonly detected mammalian order. We detected primate DNA from 71/240 (29.6%) of all flies collected, with Black Spider Monkey *Ateles chamek* detected from 40/240 flies (16.67%) and Bolivian Squirrel Monkey *Saimiri boliviensis* detected from 31/240 flies (12.92%; Table 3). Lowland Tapir *Tapirus terrestris* was the third most detected species and was detected from 14/240 flies (5.83%; Table 3).

Table 3. Summary of the number and percentage of flies collected containing DNA from each presumed species from metabarcoding of DNA from carrion flies collected from Los Amigos Biological Station, Madre de Dios, Peru.

Presumed species	Common name	number of flies with DNA	percent of all flies containing vertebrate DNA	percent of all flies collected	number of sites detected
Ateles chamek	Black Spider Monkey	40	38%	16.67%	20
Saimiri boliviensis	Bolivian Squirrel Monkey	31	29%	12.92%	15
Tapirus terrestris	Brazilian Tapir	14	13%	5.83%	10
Nasua nasua	South American Coati	13	12%	5.42%	11
Alouatta seniculus	Red Howler Monkey	12	11%	5.00%	8
Cebuella pygmaea	Eastern Pygmy Marmoset	10	9%	4.17%	7
Dicotyles tajacu	Collared Peccary	6	6%	2.50%	5
Mazama americana	Red Brocket Deer	6	6%	2.50%	5
Oligoryzomys sp.	Pygmy Rice Rat	5	5%	2.08%	5
Sapajus apella	Tufted Capuchin	4	4%	1.67%	4
Aotus nigriceps	Black-headed Night Monkey	3	3%	1.25%	2
Cebus cuscinus	Shock-headed Capuchin	3	3%	1.25%	3
Puma concolor	Puma	3	3%	1.25%	3
Bassaricyon alleni	Eastern Lowland Olingo	2	2%	0.83%	2
Mesomys hispidus	Spiny Tree Rat	2	2%	0.83%	2
Passalites nemorivagus	Amazonian Brown Brocket	2	2%	0.83%	2
Potos flavus	Kinkajou	2	2%	0.83%	2
Tayassu pecari	White-lipped Peccary	2	2%	0.83%	2
Artibeus planirostris	Flat-faced Fruit-eating Bat	1	1%	0.42%	1
Coendou sp.	Prehensile-tailed Porcupine	1	1%	0.42%	1
Cuniculus paca	Lowland Paca	1	1%	0.42%	1
Dasypus sp.	Naked-tailed Armadillo	1	1%	0.42%	1
Dendropsophus koechlini	Koechlin's Treefrog	1	1%	0.42%	1
Didelphis marsupialis	Common Opossum	1	1%	0.42%	1
Eira barbara	Tyra	1	1%	0.42%	1
Leontocebus weddelli	Weddell's Saddle-back Tamarin	1	1%	0.42%	1
Leptodactylus stenodema	San Jose White-lipped Frog	1	1%	0.42%	1
Molossus molossus	Velvety Free-tailed Bat	1	1%	0.42%	1
Scinax ictericus	Tree frog species	1	1%	0.42%	1
Uroderma bilobatum	Tent-making Bat	1	1%	0.42%	1

Pooling strategies

Template pooling (pooling of buffer prior to initial PCR), resulted in detection of a mean of 2.25 (range 0-5) species per site (n = 10 flies per pool) compared to a mean of 4.96 (range 1-8) species per site from individual flies. Template pooling detected a mean of 4.0 (range 1-7) vertebrate species per trail segment (n = 40 flies per pool) compared to a mean of 12 species per trail segment from individual flies (Table 4, Suppl. material 1: table S1). Template pooling detected the same species detected from individual flies at just 1/24 sites (4.2%), and at 0/6 trail segments.

PCR product pooling (pooling of PCR product after initial PCR from individual flies and prior to indexing), resulted in detection of a mean of 5.04 (range 1-8) species per site (n = 10 flies per pool), just slightly more than the mean number of species detected from individual flies (4.96 species per site; Table 4, Suppl. material 1: table S1). At two sites, individual flies detected one more species

Table 4. Number of vertebrate species detected by site from individual flies, and two pooling methods, from DNA metabarcoding of carrion flies from Los Amigos Biological Station, Madre de Dios, Peru.

Site	Number of species detected individual flies	Number of species detected template pools	Number of species detected PCR product pools
Site 1	6	1	6
Site 2	1	0	1
Site 3	3	1	4
Site 4	3	0	3
Site 5	5	5	5
Site 6	5	4	7
Site 7	5	3	5
Site 8	3	1	3
Site 9	4	3	4
Site 10	6	4	7
Site 11	7	2	6
Site 12	8	2	8
Site 13	5	2	4
Site 14	6	2	6
Site 15	6	4	6
Site 16	3	1	3
Site 17	3	0	3
Site 18	6	4	6
Site 19	4	1	4
Site 20	5	4	5
Site 21	6	1	6
Site 22	7	6	8
Site 23	8	2	8
Site 24	3	1	3
Mean number of species detected per site	**4.96**	**2.25**	**5.04**
Total number of species detected	**29**	**17**	**29**

than the PCR product pool. At the 22 remaining sites, the PCR pool detected the same species detected by individual flies. At three sites, the PCR product pool detected one additional species not detected by individual flies, and at one site, the PCR product pool detected two additional species. PCR product pooling detected a mean of 12.33 species per trial segment (n = 40 flies per pool), slightly higher than the mean number of species detected from individual flies (12 species). This method detected a mean of 17 species per day (n = 80 flies per pool), slightly higher than the mean number of species detected from individual flies (16.67 species; Suppl. material 1: table S1).

After rarifying read-depth from PCR product pool samples to match depth of template pool samples, results remained nearly identical to the results from the non-rarified dataset. Two exceptions were noted: at one site, we detected *Cebuella niveiventris* from the non-rarified dataset, but not from the rarified dataset, and at another site we detected *Dendropsophus koechlini* from the non-rarified dataset, but not from the rarified dataset. Otherwise, the results were identical between the rarified and non-rarified datasets.

Discussion

Our results demonstrate that carrion fly-derived iDNA is an efficient method for surveying mammal species from tropical forests. Twenty-seven unique mammalian taxa from 9 orders and 18 families plus four anuran taxa from three families were detected from just 240 carrion flies collected over the course of just 3 days. This method was highly effective at detecting arboreal species which are often difficult to detect with camera traps, including eight primates, among them the near-threatened Shock-headed Capuchin *Cebus cuscinus*, the vulnerable Eastern Pygmy Marmoset *Cebuella niveiventris*, and the endangered Black Spider Monkey *Ateles chamek* (IUCN 2025).

A key methodological advancement was the use of an extraction-free, direct PCR approach to target DNA left behind by carrion flies in collection tubes. While we are not the first to use this direct PCR approach (see Srivathsan et al. 2023), we found that this innovative method offers significant advantages. It not only streamlines fieldwork but also reduces laboratory costs by eliminating the labor-intensive DNA extraction step. Previous approaches for carrion fly metabarcoding required macerating whole flies into a slurry for DNA extraction (Calvignac-Spencer et al. 2013; Rodgers et al. 2017) or dissecting fly digestive tracts (Lee et al. 2016), processes that demand substantial effort. In contrast, the extraction-free method uses a simple buffer added to the tubes that previously contained carrion flies as DNA template in PCR, bypassing the need for maceration or dissecting of flies followed by DNA extraction. This method saves significant time and cost by completely eliminating the DNA extraction step, which is frequently the most time-consuming part of iDNA workflows. DNA extraction kits for environmental samples typically cost $3-$8 USD per sample, and thus even for small studies with hundreds of flies such as this one, savings can quickly exceed $1000 USD. Additionally, typical extraction workflows can take 2-4 hours per 24 samples, so extraction from hundreds or thousands of flies can take days or even weeks. Moreover, flies could be released alive after depositing DNA into the tubes (although ours were preserved for taxonomic identification), making this method potentially even less invasive, with minimal impact on fly populations. This extraction-free, direct PCR approach significantly improves the speed and efficiency of iDNA techniques for surveying and monitoring vertebrate biodiversity.

Although the extraction-free direct PCR approach saved considerable cost and effort, and still detected many taxa, it is possible that it could have limitations compared to traditional approaches involving maceration or dissection of flies followed by formal DNA extraction. Extraction of DNA from the entire digestive tract could potentially detect a greater number of species by amplifying DNA from all material ingested by the fly. By comparison, our extraction-free approach targets DNA present on the external surface of flies regurgitated or deposited onto the inside of tubes. Consequently, species detections may be limited to more recent feeding events. We did observe flies placing their probosces on the side of tubes and leaving behind liquid digestive fluid, which we hypothesize is the main source of vertebrate DNA in our study, as opposed to residual mammal DNA from the body surface of flies. Excrement from flies could also be a source of DNA.

With the extraction-free approach, we detected wild vertebrate DNA from 44.2% of flies collected, which is higher than most other carrion fly iDNA studies using formal DNA extraction (Calvignac-Spencer et al. 2013; Hoffmann et al. 2016; Lee et al. 2015; Owings et al. 2019). We are aware of just one study using formal DNA extraction (Schubert et al. 2015) that reported a higher percentage of flies with vertebrate DNA (52%). To our knowledge the highest reported percentage of fly samples containing vertebrate DNA (62%) comes from Srivathsan et al., (2023), which, like our study, used extraction-free direct PCR. We also detected multiple taxa from 43 individual fly samples, and as many as five species from a single fly sample. In total, we detected 31 wild vertebrate taxa with our extraction-free approach. We are aware of just two other carrion fly iDNA studies that detected a greater number of species from carrion fly metabarcoding. Gogarten et al. (2020) detected 48 vertebrate species from 784 flies collected from multiple sites across four African countries with the same 16S loci we used. Massey et al. (2022) detected 66 vertebrate taxa from 1759 fly samples collected from multiple sites in Brazil, using the same 16S marker, plus a 12S marker and a COI marker capable of detecting DNA from mammals as well as non-mammalian vertebrates. As we detected 31 taxa from just 240 fly samples with one marker, our study detected a greater number of vertebrate taxa per fly than either of these studies, and from a much smaller geographical area. Thus, although we did not directly compare extraction-free direct PCR with maceration and formal DNA extraction, our results clearly demonstrate that extraction-free direct PCR methods are not only efficient, but also highly effective for vertebrate monitoring from carrion flies and are on par with methods using maceration and formal DNA extraction.

Another goal of this study was to improve the scalability of using carrion flies for large-scale surveys of vertebrate diversity. Although the current study used just 240 carrion flies collected over three days, future studies may require thousands of flies collected over much larger areas and timescales. To this end, we tested a method for collecting carrion flies by netting them directly off a bag of bait rather than using bottle-traps as has been done in some past studies (Rodgers et al. 2017). We were able to collect 10 flies from each site in approximately 15-20 minutes. This method is advantageous compared to trapping with bottle-traps for two reasons. First, it only requires visiting each site once to collect flies, whereas trapping requires moving traps from location to location, and then visiting the traps the following day to collect flies. Second, it requires very little equipment, just a good insect net, and a plastic bag full of bait. Thus, this simple sampling method may be preferable for large-scale surveys spanning many locations, as each site can be visited just once for a short period of time without the need to return to collect traps. We note, however, that capture rates, and thus optimal methods, may vary considerably by site, weather, season, or day, and in some cases fly traps may still be preferable.

Pooling strategies

Two different strategies were evaluated for metabarcoding of pooled samples containing DNA from multiple carrion flies: template pooling, and PCR product pooling. This evaluation provides important insight because for larger-scale studies with potentially thousands of individual flies, it would be prohibitively expensive to sequence each fly individually. The ability to pool flies collected

from the same site prior to sequencing is advantageous to decrease sequencing cost while still maintaining the ability to differentiate between sites. Pooling of multiple flies, however, may lead to missed species detections due to sample dilution (Broadhurst et al. 2025; Rodgers et al. 2017; Sato et al. 2017). For instance, Rodgers et al. (2017) pooled 16 macerated flies into each DNA extraction but also sequenced some individual flies for comparison. They found that from 24 flies extracted individually and added to pools, four total mammal species were detected from pools, whereas 10 total mammal species were detected from the same 24 flies when extracted individually. To further investigate the effects of pooling, we tested two methods for pooling fly samples that were collected at the same site and compared results of these pooling methods with results from individual flies. Template pooling resulted in the loss of many species compared to sequencing of individual flies, likely due to sample dilution such that template DNA from all species was not present in the aliquot added to PCR, and thus not amplified.

Conversely, PCR product pooling after conducting an initial PCR from individual fly samples, but prior to sample indexing, performed exceptionally well. Minor discrepancies were noted, but overall, the results from PCR product pooling were nearly identical to results obtained from individual flies (Table 4, Suppl. material 1: table S1). This post-PCR pooling method is more time-consuming and costly than template pooling, as an initial PCR must be conducted from each fly sample. However, it still allows many more fly samples to be multiplexed and sequenced in a single Next-Generation Sequencing run, making it a feasible method for large-scale studies with large numbers of samples. Initial sequencing depth was approximately nine times higher for the PCR product pooling method versus the template pooling method. However, rarefaction analysis indicated that read-count differences did not account for the differences in species detection observed between the two pooling strategies. Thus, the PCR product pooling strategy was clearly preferable, as it performed nearly identically to sequencing of individual flies, without loss of species detection, whereas the template pooling method resulted in the non-detection of many species. It is possible, however, that results from template pooling may have improved with greater sequencing depth.

We found that, in a few cases, species were detected in pooled samples that were not detected from individual flies. PCRs from individual flies were run with just 2 µL (5%) of the total buffer collected from each tube as template, so it is likely that in some cases, DNA from a species was present in the tube but was not present in the template that was added to PCR. For example, the Brown egg frog *Ctenophryne geayi* was detected from template pooling but was not detected from individual flies or by PCR product pooling (Suppl. material 1: table S1). Only 49 reads of this species were detected from a single pooled sample indicating that the DNA of this species was likely at low concentration. Running a greater number of PCR replicates per sample would decrease the chance of missing species with very little DNA present; however, this approach would substantially increase cost. Another potential approach would be to pool buffer and then concentrate the pooled buffer such that the entire pool could be included as template in a single PCR. Alternatively, multiple flies could be pooled in the same tube when collected in the field for downstream extraction-free PCR. These pooling approaches could potentially be more cost effective than conducting PCR on each individual fly and should be investigated in future research.

Taxonomic assignment

We tested two different methods for taxonomic assignment of OTUs derived from carrion fly metabarcoding, BLAST (Altschul et al. 1990) and a naïve Bayes classifier using scikit-learn (Pedregosa et al. 2011) implemented within the QIIME2 environment. Both methods provided consistent assignments for nearly all OTUs with a few exceptions (Table 2). In one case the naïve Bayes classifier classified an OTU at a relatively low confidence (0.86) to the genus *Odocoileus*, a genus of cervid that is not known from our study area. BLAST returned a hit for *Mazama americana*, a cervid species that is known from our study area, with a 100% match to the reference sequence. Thus, we presumed this OTU originated from *M. americana* (Table 2). Interestingly, however, BLAST also returned hits that were a 100% match to *Odocoileus virginianus*, *Odocoileus hemionus*, *Mazama nana*, *Mazama rufa*, and *Mazama bororo*, none of which occur in our study area. Thus, while we still conclude this OTU likely originated from *M. americana*, the 16S marker alone cannot differentiate these species. In another case, both methods identified one OTU as *Passalites nemorivagus*, the Amazonian brown brocket, a species which is known from our study area (Table 2). This OTU was a 98.9% match to a reference sequence from *P. nemorivagus* but was also a 98.9% match to reference sequences from *Ozotoceros bezoarticus*, a South American cervid species not known from our study area, and *Rangifer tarandus* or Reindeer. Thus, it is likely that the 16S amplicon used in this study may have relatively poor taxonomic power for distinguishing cervid species. These cases emphasize the fact that, in many instances, prior knowledge of the local wildlife community can aid in the correct assignment of taxa at the species level. Additionally, using multiple taxonomic classifiers can assist the assignment of sequences to the correct species. In the case of the OTU ultimately assigned to *M. americana,* use of only the naïve Bayes classifier would have led to an incorrect assignment. We also potentially detected two mammal species from our study area that lack reference sequences for the 16S amplicon used in our study: *Tayassu pecari* and *Eira barbara.* As these species have wide Neotropical distributions and are of conservation concern in some areas, generating 16S reference sequences for these species should be a priority so that they can be accurately identified in future eDNA/iDNA studies.

In two instances, sequences were assigned to primate species that are not known from our study area. One OTU was assigned with high probability by both classifiers as the capuchin monkey species *Cebus unicolor. C. unicolor* is not known from our study area, however a closely related species, *C. cuscinus* is. *C. cuscinus* and *C. unicolor* were historically considered subspecies of *C. albifrons;* however, they have since been split into separate species (Boubli et al. 2012). Although this split was based in part on mitochondrial genetics, it appears likely that *C. cuscinus* and *C. unicolor* are genetically identical at our short 16S mitochondrial locus, as the sequence we found was a 100% match to a *C. unicolor* sequence. No *C. cuscinus* reference sequences are available, but we presume that the sequence we detected is from *C. cuscinus* as it is the only *Cebus* species known from our study area. Both classifiers also assigned an OTU to the genus *Cebuella* or pygmy marmoset. Pygmy marmosets are not known to be present at Los Amigos, however, the eastern pygmy marmoset *Cebuella niveiventris* is known from the region. Both classifiers identified this

sequence with somewhat lower confidence, the naïve Bayes classifier only identified the sequence to genus level at 0.974 confidence, and BLAST had a top hit match percentage of just 93.4% with the sister species *Cebuella pygmaea*. Thus, it is possible that this classification is artifactual. However, it would seem unlikely that a sequence would be artificially assigned to a species known from the region just by chance. This sequence was also found in 10 individual flies, making it the 6th most commonly detected taxa in our study, which is peculiar for a species that has never been observed at our study site. One possibility is that a dead marmoset floated downriver from a population upriver and was being actively consumed by flies when our sampling occurred, which would explain why the sequence was detected from a relatively large number of flies. Because this detection was unexpected, and confidence in the assignment was lower than for most other species, it should be treated with some uncertainty.

Conclusion

This study demonstrates the effectiveness of using carrion fly-derived iDNA in mammalian biodiversity surveys in tropical rainforest. We detected 27 unique mammal species plus four anuran species from just 240 carrion flies. A key methodological advancement was the extraction-free, direct PCR approach we used, which simplifies both field and lab work, significantly reducing time and costs while maintaining robust species detection. This method offers a non-invasive and efficient approach for rainforest mammal monitoring and appears to be particularly valuable for the detection of arboreal primates. Additionally, we investigated methods for scaling-up carrion fly metabarcoding surveys, including a more efficient collection strategy, as well as pooling methods for cost-effective sequencing. Pooling PCR products after initial amplification from individual flies, but prior to indexing, proved highly effective, nearly perfectly matching results from sequencing of individual flies without sacrificing species detection accuracy, making it a viable method for future large-scale iDNA surveys. Our findings will contribute to the efficiency and practicability of using iDNA metabarcoding for future biodiversity monitoring and conservation of mammals in tropical forests.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

The following AI tools were used in the preparation of this manuscript (select all that apply): Description: AI assisted tools were used in some basic editing while writing this manuscript.

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Author contributions

Conceptualization: TWWR, AF. Data curation: EMRG, TWWR. Formal analysis: TWWR, CCYX. Funding acquisition: AF. Investigation: EMRG, TWWR, AB, RC, EH. Methodology: AF, TWWR. Project administration: TWWR, AF. Resources: KEM, AF. Supervision: KEM, AF. Writing - original draft: TWWR. Writing - review and editing: EMRG, AB, KEM, CCYX, AF.

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Data availability

All metabarcoding sequence data can be found on the NCBI Sequence Read Archive under BioProject PRJNA132248. Biodiversity occurrence data can be found on GBIF at https://doi.org/10.15468/29jx7n.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Boessenkool S, Epp LS, Haile J, Bellemain E, Edwards M, Coissac E, Willerslev E, Brochmann C (2012) Blocking human contaminant DNA during PCR allows amplification of rare mammal species from sedimentary ancient DNA. Molecular Ecology 21: 1806–1815. https://doi.org/10.1111/j.1365-294X.2011.05306.x

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Yu DW, de Bruyn M (2014) Environmental DNA for wildlife biology and biodiversity monitoring. Trends in Ecology & Evolution 29: 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang K Bin, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL,

Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37: 852–857. https://doi.org/10.1038/s41587-019-0209-9

Boubli JP, Rylands AB, Farias IP, Alfaro ME, Alfaro JL (2012) Cebus Phylogenetic Relationships: A Preliminary Reassessment of the Diversity of the Untufted Capuchin Monkeys. American Journal of Primatology 74: 381–393. https://doi.org/10.1002/ajp.21998

Broadhurst HA, Sales NG, Raynor R, Howe C, Ochu E, Lambin X, Sutherland CS, McDevitt AD (2025) From Water to Land: A Review on the Applications of Environmental DNA and Invertebrate-Derived DNA for Monitoring Terrestrial and Semi-Aquatic Mammals. Mammal Review 55: e70006. https://doi.org/10.1111/mam.70006

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13: 581. https://doi.org/10.1038/nmeth.3869

Calvignac-Spencer S, Merkel K, Kutzner N, Kühl H, Boesch C, Kappeler PM, Metzger S, Schubert G, Leendertz FH (2013) Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. Molecular Ecology 22: 915–924. https://doi.org/10.1111/mec.12183

Drinkwater R, Williamson J, Clare EL, Chung AYC, Rossiter SJ, Slade E (2021) Dung beetles as samplers of mammals in Malaysian Borneo—a test of high throughput metabarcoding of iDNA. Baxter-Gilbert J (Ed.). PeerJ 9: e11897. https://doi.org/10.7717/peerj.11897

Gogarten JF, Hoffmann C, Arandjelovic M, Sachse A, Merkel K, Dieguez P, Agbor A, Angedakin S, Brazzola G, Jones S, Langergraber KE, Lee K, Marrocoli S, Murai M, Sommer V, Kühl H, Leendertz FH, Calvignac-Spencer S (2020) Fly-derived DNA and camera traps are complementary tools for assessing mammalian biodiversity. Environmental DNA 2: 63–76. https://doi.org/10.1002/edn3.46

Hoffmann C, Stockhausen M, Merkel K, Calvignac-Spencer S, Leendertz FH (2016) Assessing the feasibility of fly based surveillance of wildlife infectious diseases. Scientific Reports 6. https://doi.org/10.1038/srep37952

IUCN (2025) The IUCN Red List of Threatened Species. Version 2025-1. https://www.iucnredlist.org [May 15, 2025]

Jahan M, Lynggaard C, Agbor A, Angedakin S, Armero A, Corogenes K, Dieguez P, Jones S, Krückemeier S, Langergraber KE, Lee KC, Murai M, Normand E, van Schijndel J, Sommer V, Wessling EG, Leendertz FH, Boesch C, Kühl HS, Arandjelovic M, Gogarten JF, Calvignac-Spencer S (2025) An Exploration of DNA Extraction Methods of Fly iDNA for Scalable Biodiversity Monitoring. Environmental DNA 7: e70215. https://doi.org/10.1002/edn3.70215

Ji Y, Baker CCM, Popescu VD, Wang J, Wu C, Wang Z, Li Y, Wang L, Hua C, Yang Z, Yang C, Xu CCY, Diana A, Wen Q, Pierce NE, Yu DW (2022) Measuring protected-area effectiveness using vertebrate distributions from leech iDNA. Nature Communications 13: 1555. https://doi.org/10.1038/s41467-022-28778-8

Lee PS, Sing KW, Wilson JJ (2015) Reading mammal diversity from flies: The persistence period of amplifiable mammal mtDNA in blowfly guts (Chrysomya megacephala) and

a new DNA mini-barcode target. PLoS ONE 10: 1–12. https://doi.org/10.1371/journal.pone.0123871

Lee P-S, Gan HM, Clements GR, Wilson J-J, Adamowicz S (2016) Field calibration of blowfly-derived DNA against traditional methods for assessing mammal diversity in tropical forests [1]. Genome 59: 1008–1022. https://doi.org/10.1139/gen-2015-0193

Leray M, Knowlton N, Machida RJ (2022) MIDORI2: A collection of quality controlled, preformatted, and regularly updated reference databases for taxonomic assignment of eukaryotic mitochondrial sequences. Environmental DNA 4: 894–907. https://doi.org/10.1002/edn3.303

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17: 10. https://doi.org/10.14806/ej.17.1.200

Massey AL, Bronzoni RV de M, da Silva DJF, Allen JM, de Lázari PR, dos Santos-Filho M, Canale GR, Bernardo CSS, Peres CA, Levi T (2022) Invertebrates for vertebrate biodiversity monitoring: Comparisons using three insect taxa as iDNA samplers. Molecular Ecology Resources 22: 962–977. https://doi.org/10.1111/1755-0998.13525

Newton JP, Allentoft ME, Bateman PW, van der Heyde M, Nevill P (2025) Targeting Terrestrial Vertebrates With eDNA: Trends, Perspectives, and Considerations for Sampling. Environmental DNA 7: e70056. https://doi.org/10.1002/edn3.70056

Owings CG, Banerjee A, Asher TMD, Gilhooly WP, Tuceryan A, Huffine M, Skaggs CL, Adebowale IM, Manicke NE, Picard CJ (2019) Female Blow Flies As Vertebrate Resource Indicators. Scientific Reports 9: 10594. https://doi.org/10.1038/s41598-019-46758-9

Parisi Dutra R, Casali D de M, Missagia RV, Gasparini GM, Perini FA, Cozzuol MA (2017) Phylogenetic Systematics of Peccaries (Tayassuidae: Artiodactyla) and a Classification of South American Tayassuids. Journal of Mammalian Evolution 24: 345–358. https://doi.org/10.1007/s10914-016-9347-8

Payne CJ, Champagne PS, O'Donnell H, Lange LR, Rushford C, Rosolie P, Rosenzweig D (2024) High mammalian diversity on the Las Piedras River tributary of Madre de Dios, Peru: an annotated list of species including comments on biogeography and regional conservation. Check List 20: 152–183. https://doi.org/10.15560/20.1.152

Pedregosa F, Michel V, Grisel O, Blondel M, Prettenhofer P, Weiss R, Vanderplas J, Cournapeau D, Pedregosa F, Varoquaux G, Gramfort A, Thirion B, Grisel O, Dubourg V, Passos A, Brucher M, Perrot andÉdouardand M, Duchesnay andÉdouard, Duchesnay Fredouard (2011) Scikit-learn: Machine Learning in Python. Journal of Machine Learning Research 12: 2825–2830. http://scikit-learn.sourceforge.net

Rodgers TW, Xu CCY, Giacalone J, Kapheim KM, Saltonstall K, Vargas M, Yu DW, Somervuo P, McMillan WO, Jansen PA (2017) Carrion fly-derived DNA metabarcoding is an effective tool for mammal surveys: Evidence from a known tropical mammal community. Molecular Ecology Resources 17: e133–e145. https://doi.org/10.1111/1755-0998.12701

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. Hrbek T (Ed.). PeerJ 4: e2584. https://doi.org/10.7717/peerj.2584

Ruppert KM, Kline RJ, Rahman MS (2019) Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Global Ecology and Conservation 17: e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Saranholi BH, França FM, Vogler AP, Barlow J, Vaz de Mello FZ, Maldaner ME, Carvalho E, Gestich CC, Howes B, Banks-Leite C, Galetti Jr PM (2024) Testing and optimizing metabarcoding of iDNA from dung beetles to sample mammals in the hyperdiverse

Neotropics. Molecular Ecology Resources 24: e13961. https://doi.org/10.1111/1755-0998.13961

Sato H, Sogo Y, Doi H, Yamanaka H (2017) Usefulness and limitations of sample pooling for environmental DNA metabarcoding of freshwater fish communities. Scientific Reports 7: 14860. https://doi.org/10.1038/s41598-017-14978-6

Schubert G, Stockhausen M, Hoffmann C, Merkel K, Vigilant L, Leendertz FH, Calvignac-Spencer S (2015) Targeted detection of mammalian species using carrion fly-derived DNA. Molecular Ecology Resources 15: 285–294. https://doi.org/10.1111/1755-0998.12306

Srivathsan A, Loh RK, Ong EJ, Lee L, Ang Y, Kutty SN, Meier R (2023) Network analysis with either Illumina or MinION reveals that detecting vertebrate species requires metabarcoding of iDNA from a diverse fly community. Molecular Ecology 32: 6418–6435. https://doi.org/10.1111/mec.16767

Taylor PG (1996) Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. Molecular Biology and Evolution 13: 283–285. https://doi.org/10.1093/oxfordjournals.molbev.a025566

Supplementary material 1

Additional information

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Data type: xlsx

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